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Nested reverse transcriptase–polymerase chain reactions targeting the messenger RNA of icl_2, *hspx*, and *rRNAP1* genes to detect viable *Mycobacterium tuberculosis* directly from clinical specimens

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ABSTRACT

There is an urgent need for a rapid and reliable test to detect actively multiplying *Mycobacterium tuberculosis* directly from clinical specimens for an early initiation of the appropriate antituberculous treatment. This study was aimed at the optimization and application of nested reverse transcriptase–PCR (nRT–PCR) targeting the messenger RNA of the icl_2, *hspx*, and *rRNAP1* genes directly from sputum specimens, and their evaluation against the culture by the BACTEC MicroMGIT mycobacterial culture system. 203 Sputum samples from clinically suspected tuberculosis patients and 30 control specimens (clinically proven viral or bacterial infections other than tuberculosis) were included in this study. The mycobacterial culture was performed by the BACTEC MicroMGIT system following the manufacturer's instructions. The primers for nRT–PCRs targeting icl_2, *hspx*, and *rRNAP1* genes were indigenously designed using the Primer-BLAST software, and optimized for sensitivity and specificity. The icl_2, *hspx*, and *rRNAP1* genes were able to pick up 63.9%, 67.2%, and 58.75%, respectively, of culture-negative sputum specimens collected from clinically suspected tuberculosis patients. However, three (1.4%) were negative for nRT–PCR, but *M. tuberculosis* culture positive. All the 30 controls were negative for culture by the BACTEC MicroMGIT method and all three nRT–PCR. The novel nRT–PCRs targeting icl_2, *hspx*, and *rRNAP1* genes developed in this study are rapid and reliable diagnostic tools to detect viable *M. tuberculosis* directly from sputum specimens. However, further study by including a larger number of sputum specimens needs to be carried out to ascertain the diagnostic utility of the novel nRT–PCRs optimized in the study.

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Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects approximately 2 billion people worldwide, and is the leading cause of mortality due to this disease. The diagnosis of TB is difficult in situations where clinical diagnosis is suggestive, but bacteriological proof is lacking. The detection of acid-fast bacilli (AFB) by conventional microscopy is simple and rapid, but lacks adequate sensitivity, whereas culture is comparatively more sensitive and specific, but the result is available after several weeks [1]. Hence, there is a need for an alternative diagnostic method, which is specific, sensitive, and rapid, particularly so when bacteriological proof of diagnosis is lacking. In recent years, the polymerase-chain-reaction (PCR) test has been found to be useful for rapid diagnosis of TB directly from clinical specimens [1].

The gene of *M. tuberculosis*, icl_2 (RV0467), encoding for isocitrate lyase was previously demonstrated to play a pivotal role in the intracellular metabolism of actively multiplying *M. tuberculosis*. Isocitrate lyase is one of the key enzymes of the glyoxylate metabolism shunt [2]. During the growth on C_2 substrates, such as fatty acids or acetate, most microorganisms employ the glyoxylate shunt as the main metabolic route for the biosynthesis of cellular materials, and survive inside the host macrophages. The *hspx* gene (Rv 2031c) encodes the α-crystalline homologue protein. It is a 16-kDa heat shock protein required for mycobacterial persistence within the macrophage [2]. Many reports state that the *hspx* gene is upregulated in macrophages during the initial stages of TB. A recent report highlighted the high levels of *hspx* messenger RNA (mRNA) in log-phase cultures of *M. tuberculosis* [3]. Experimental evidence also indicated that the *hspx* gene level is elevated during the growth of *M. tuberculosis* in cultured macrophages [3]. The *rRNAP1* gene (X58890.1) of *M. tuberculosis* is a noncoding ribosomal promoter region, mainly involved in the transcription of *M. tuberculosis*. The rrnA-P1, one of the five rrn promoters in mycobacteria, has been described as a novel target to detect *M. tuberculosis* [4]. The present study is focused on targeting the mRNA of the icl_2, *hspx*, and *rRNAP1* genes to detect the presence of actively multiplying *M. tuberculosis* directly on sputum specimens from clinically suspected TB patients for an early initiation of the antituberculous treatment.

Materials and methods

The study was carried out at Larsen and Toubro Microbiology Research Centre, Vision Research Foundation, Chennai, India. The study (study code: 93-2007-P) was approved by the Research Cell and Ethics Sub-Committee (Institutional Review Board, Vision Research Foundation on March 21, 2008).

Sputum collection

A total of 203 sputum specimens from clinically suspected TB patients attending the out patient department (OPD) at the Institute of Thoracic Medicine, Chetput, Chennai, India, and 30 control sputum specimens (from clinically proven non-TB patients) collected from March 2013 to July 2013 were included in this study. The informed consent was obtained from all the patients included in the study. The sputum specimens were collected in new vials coated with sterile 0.1% diethyl pyrocarbonate to prevent RNA degradation. The collected sputum specimens were transported to the laboratory within an hour of collection.

Processing of sputum specimens

Direct smear study

Ziehl–Neelsen staining was performed on all direct and concentrated smears of 203 sputum specimens to look for the presence of AFB according to the Revised National Tuberculosis Control Programme guidelines.

Culture for isolation of ***M. tuberculosis***

The sputum samples were decontaminated using the modified Petroff's method (*n*-acetyl-L-cysteine/sodium-hydroxide method) following the instructions given in the BACTEC manual (Becton Dickinson diagnostics, New Jersey, United states), and inoculated in MicroMGIT tubes. The inoculated tubes were incubated at 37 °C for 42 days, and readings were taken every day.

RNA extraction and complementary DNA conversion

RNA extraction was carried out using the TRIzol method followed by complementary DNA (cDNA) conversion (Table 1) of the RNA using the Sensiscript reverse-transcription kit (Qiagen, Valencia, California).

Performance of nested reverse transcriptase–PCRs targeting the icl_2, hspx, and rRNAP1 genes

After cDNA conversion by the Sensiscript reverse-transcription kit (ABI Biosystems, Waltham, Massachusetts, USA), the nested reverse transcriptase–PCRs (nRT–PCRs) targeting the icl_2, *hspx*, and *rRNAP1* genes were performed. The primer sets (both inner and outer sets of primers) targeting the mRNA of the icl_2, *hspx*, and *rRNAP1* genes were designed indigenously using the Primer-BLAST software from the National Center for Biotechnology Information (NCBI). The primers designed are given in Table 2. The primers were checked by BLAST search and matching with the sequence available in NCBI for specificity.

Table 1 – Complementary DNA conversion protocol.

Reagents	Volume (μL)	Incubation
dNTP mix	1	37 °C for 1 h
RT buffer	1	
Oligo-dt	1	
Milli-Q	11.5	
RT enzyme	0.5	
RNA	5	

Note: dNTP = deoxynucleotide triphosphate; dt = deoxythymine; RT = reverse transcription.

Table 2 – Details of primer sequence, thermal profile, and amplicon size used to standardize nested reverse transcriptase–polymerase chain reactions.

Primer sequence (5′–3′) (indigenously designed primers)	Nucleotide positions of the primers within the genes	Thermal profile	No. of cycles	Amplicon size (bp)
icl_2 gene (total length: 1287 base pairs)				
First-round primer sets	255–861	94 °C for 1 min	30	607
ICLP1F:GAAGGCCATCTACCTGTCGG		64 °C for 1 min		
ICLPIR:ACCGGTCTCCATCCAGATCA		72 °C for 1 min		
Second-round primer sets	251–677	94 °C for 1 min	25	427
ICLP5F:GCCTGAAGGCCATCTACCTG		64 °C for 1 min		
ICLP5R:ATCACCACCGTGGGAACATC		72 °C for 1 min		
hspx gene (total length: 635 base pairs)				
First-round primer sets	145–400	94 °C for 1 min	30	261
HSPP3F: CGGCTGGAAGACGAGATGAA		64 °C for 1 min		
HSPP3R: CGCCACCGACACAGTAAGAA		72 °C for 1 min		
Second-round primer sets	440–600	94 °C for 1 min	25	161
HSPP1F: TTATGGTCCGCGATGGTCAG		64 °C for 1 min		
HSPP1R: AATGCCCTTGTCGTAGGTGG		72 °C for 1 min		
rRNAP1 gene (total length: 478 base pairs)				
First-round primer sets	53–459	98 °C for 10 s	35	407
RRNAP1F5: TCACGGAGAACGTGTTCGAG		60 °C for 30 s		
RRNAP1R5: ACAACACGCTTGCTTGTTTCC		72 °C for 1 min		
Second-round primer sets	291–456	98 °C for 10 s	25	166
RRNAP1F4: CGTGGAGAACCTGGTGAGTC		62 °C for 30 s		
RRNAP1R4: ACACGCTTGCTTGTTTCCG		72 °C for 30 s		

Cocktail preparation for nRT–PCR targeting the mRNA of the icl_2 and hspx genes

A 25-μL PCR reaction includes 22 μL of cocktail consisting of 200 μM of each deoxynucleotide triphosphate (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate), 1 μM of each primer set, and 10 × buffers (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), and 1 unit of *Taq* DNA polymerase was aliquoted into each vial. The negative control was placed inside the PCR machine, and 3 μL of cDNA was added into the respective labeled vials, and 3 μL of positive-control cDNA (H37Rv) was pipetted into the vial labeled as positive control. For the second round, 2.5 μL of the amplified product was transferred to the second-round cocktail.

Cocktail preparation for nRT–PCR targeting the mRNA of the rRNAP1 gene

A 25-μL PCR reaction, including 12.5 μL of Emerald GT Master Mix (Takara Bio. inc., Shiga, Japan), 10 μL of GT water, and 1 μM of each primer set, was prepared. The negative control was placed inside the PCR machine, and 1.5 μL of cDNA was added into the respective labeled vials, and 1 μL of positive-control cDNA (H37Rv) was pipetted into the vial labeled as the positive control. For the second round, 2 μL of the amplified product was transferred to the second-round cocktail (Table 2).

Determination of analytical sensitivity of nRT–PCRs targeting the icl_2, hspx, and rRNAP1 genes

The analytical sensitivity of the nRT–PCRs was performed by serial tenfold dilutions of cDNA. Briefly, 10 Eppendorf (Hamburg, Germany) vials (0.5 mL) were taken and labeled from 10^{-1} to 10^{-10} dilution. Then, 45 μL of sterile milliQ water (Merck Millipore, Ontario, Canada) was taken in each vial, and 5 μL of freshly extracted cDNA was added to the first vial. It was mixed well, and 5 μL was transferred to the next vial. Serial dilutions were performed until the last vial, and 5 μL was discarded from the last dilutions followed by the performance of nRT–PCRs for determining the sensitivity of the primers.

Determination of specificity of nRT–PCRs targeting the icl_2, hspx, and rRNAP1 genes

The specificity of the primers was tested by amplifying the cDNA from the following strains of mycobacterial species: *M. tuberculosis* H37Rv and H37Ra, *Mycobacterium bovis*, *Mycobacterium intracellulare* (American Type Culture Collection [ATCC] 1403), *Mycobacterium kansasii* (ATCC 1201), *Mycobacterium xenopi* (ATCC 1432), *Mycobacterium gordonae*, *Mycobacterium fortuitum* (ATCC 1529), *Mycobacterium chelonae* (ATCC 1524), *Mycobacterium abscessus* (laboratory isolate), *Mycobacterium smegmatis* (ATCC 607), *Mycobacterium phlei*, *Mycobacterium thermoresistibile*, and *Mycobacterium flavescens* obtained from the National Institute for Research in Tuberculosis Research Centre, Indian Council of Medical research (ICMR) unit, Chennai, India.

Detection of amplified products

After the nRT–PCR, 10 μL of the amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5 μg/mL ethidium bromide for visualization by UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

Results

Direct smear microscopy

Out of the 203 sputum specimens from clinically suspected TB patients, 24 (11.8%) showed the presence of AFB by Ziehl–Neelsen stain, and 179 were negative.

Mycobacterial culture by the MicroMGIT BACTEC method

Out of the 203 sputum samples collected from clinically suspected TB patients, 48 (23.6%) sputum samples were culture positive and 155 (76.3%) were culture negative. All *M. tuberculosis* isolates (48) were confirmed by PCR targeting the *MPB64* gene, *IS6110* [5] region, and TBc ID kit (BD Diagnostics, New Jersey, USA). All the 30 control sputum specimens were negative for culture by the MicroMGIT BACTEC method.

Results of nRT–PCRs targeting the mRNA of the icl_2 gene on sputum samples

The designed primers were found specific and sensitive to detect 64.2 pg for the detection of *M. tuberculosis* H37Rv cDNA only. Out of the 203 sputum specimens from clinically suspected TB patients, 111 (54.6%) were positive for nRT–PCR targeting the icl_2 gene. Among these, 24 (21.6%) were AFB smear positive, 42 (37.8%) were culture positive, and 69 (62.1%) were culture negative by the MicroMGIT BACTEC method, but six culture-positive sputum specimens were negative for nRT–PCR targeting the icl_2 gene. All the 30 controls were negative for nRT–PCR targeting the icl_2 gene.

Results of nRT–PCRs targeting the mRNA of the hspx gene on sputum specimens

The designed primers were found specific and sensitive to detect 0.642 pg for the detection of *M. tuberculosis* H37Rv cDNA only. Out of the 203 sputum specimens, 107 (52.7%) were positive for nRT–PCR targeting the *hspx* gene. Among the 107 *hspx* gene-positive clinical specimens, 24 (22.4%) were AFB smear positive, 38 (35.5%) were culture positive, and 69 (64.4%) were culture negative by the MicroMGIT BACTEC method, but 10 culture-positive sputum specimens were negative for nRT–PCR targeting the *hspx* gene. All the 30 controls were negative for nRT–PCR targeting the *hspx* gene.

Results of nRT–PCRs targeting the mRNA of the rRNAP1 gene on sputum specimens

The designed primers were found specific and sensitive to detect 0.642 pg for the detection of *M. tuberculosis* H37Rv cDNA only. Out of the 203 sputum specimens, 80 (39.1%) were positive for nRT–PCR targeting the *rRNAP1* gene. Among these, 24 (30%) were AFB smear positive, 35 (43.75%) were culture positive, and 45 (56.25%) were culture negative, but 13 culture-positive sputum specimens were negative for nRT–PCR targeting the *rRNAP1* gene. All the 30 controls were negative for nRT–PCR targeting the *rRNAP1* gene (Table 3). The gel electrophoretograms of amplified products of

Table 3 – Evaluation of nested reverse transcriptase–polymerase chain reactions targeting the icl_2, *hspx*, and *rRNAP1* gene results against culture-positive *Mycobacterium tuberculosis* isolates by the BACTEC method in 203 sputum specimens.

Categories of positivity	nRT–PCR positive results						nRT–PCRs targeting the icl_2, *hspx*, & *rRNAP1* genes negative
	icl_2 gene alone	*hspx* gene alone	*rRNAP1* gene alone	icl_2 & *hspx* genes	icl_2 & *rRNAP1* genes	*hspx* & *rRNAP1* genes	icl_2, *hspx*, & *rRNAP1* genes
No. of specimens clinically suspected to be tuberculosis (203)	20 (9.8%)	11 (5.4%)	6 (2.9%)	26 (12.8%)	4 (1.9%)	9 (4.4%)	65 (32%)	62 (31%)
M. tuberculosis culture positive by the BACTEC system (48)	6 (2.9%)	2 (0.9%)	1 (0.4%)	2 (1.9%)	1 (0.4%)	Nil	33 (16.2%)	3 (1.4%)

Note: All the 30 controls were negative for both mycobacterial culture by the BACTEC method and nRT–PCRs targeting icl_2, *hspx*, and *rRNAP1* genes. nRT–PCR = nested reverse transcriptase–polymerase chain reaction.

nRT–PCR targeting the icl_2, *hspx*, and *rRNAP1* genes are given in Fig. 1.

Discussion

TB has been reported to be the major cause of death among infectious diseases. The molecular markers of *M. tuberculosis* viability are attractive, since results are rapid and there is a potential for great analytical sensitivity [2]. Molecular diagnostic methods, like the reverse transcriptase–PCR, which detects actively multiplying *M. tuberculosis* directly from sputum specimens, help the clinicians to initiate proper antituberculous treatment as early as possible, so that the rate of spreading of TB can be controlled.

The main objective of this study was to standardize nRT–PCRs targeting the mRNA of the icl_2, *hspx*, and *rRNAP1* genes to efficiently detect actively multiplying *M. tuberculosis* directly from clinical specimens, which paves the way for the early initiation of appropriate anti-TB drugs.

Previous studies by Dietze et al. [6] have demonstrated that DNA assays are not useful in monitoring response to therapy, since *M. tuberculosis* DNA persists well beyond the time points that cultures are positive. In another study, Li et al. [2] have compared four different *M. tuberculosis* mRNA targets (85B, *hspx*, icl_2, and *rRNAP1* genes) in the context of an early bactericidal-activity study by comparing isoniazid with three newer fluoroquinolones. They found that the icl_2 mRNA was determined to be the best marker based on high levels of expression in sputum and strong correlation with colony-forming-unit counts, both at baseline and during the 7 days of isoniazid monotherapy. Muñoz-Elías and McKinney [7] demonstrated that the icl_2 gene coding for isocitrate lyase is essential for *M. tuberculosis* growth and persistence in macrophages. In a study by Bwanga, Hoffner, Haile, and Joloba [8], the transcription of the *hspx* gene isolated from an actively replicating culture of *M. tuberculosis* was assessed, and they found that the expression of *hspx* is enhanced in the actively growing culture at 7 days and 10 days of culture of *M. tuberculosis*.

In the present study, nRT–PCRs targeting the mRNA of the icl_2, *hspx*, and *rRNAP1* genes were optimized using indigenously designed primers using the Primer-BLAST software from NCBI, and applied on 203 sputum specimens and 30 control specimens. The nRT–PCRs targeting the icl_2 and *hspx* genes have almost equal positivity with 54.1% and 52.7%, respectively, to detect from clinically suspected TB patients than the *rRNAP1* gene with 39.1% positivity. In co-relation of nRT–PCR results with that of the BACTEC culture, the icl_2, *hspx*, and *rRNAP1* genes were able to pick up 63.9%, 67.2%, and 58.75% more culture-negative sputum specimens, respectively. None of the controls (30) were positive for both mycobacterial culture by the BACTEC method and the nRT–PCRs targeting the icl_2, *hspx*, and *rRNAP1* genes. Even though in some cases, nRT–PCR was negative for any one of the optimized genes, the remaining three genes were able to pick up the viable *M. tuberculosis* from the clinical specimens. The

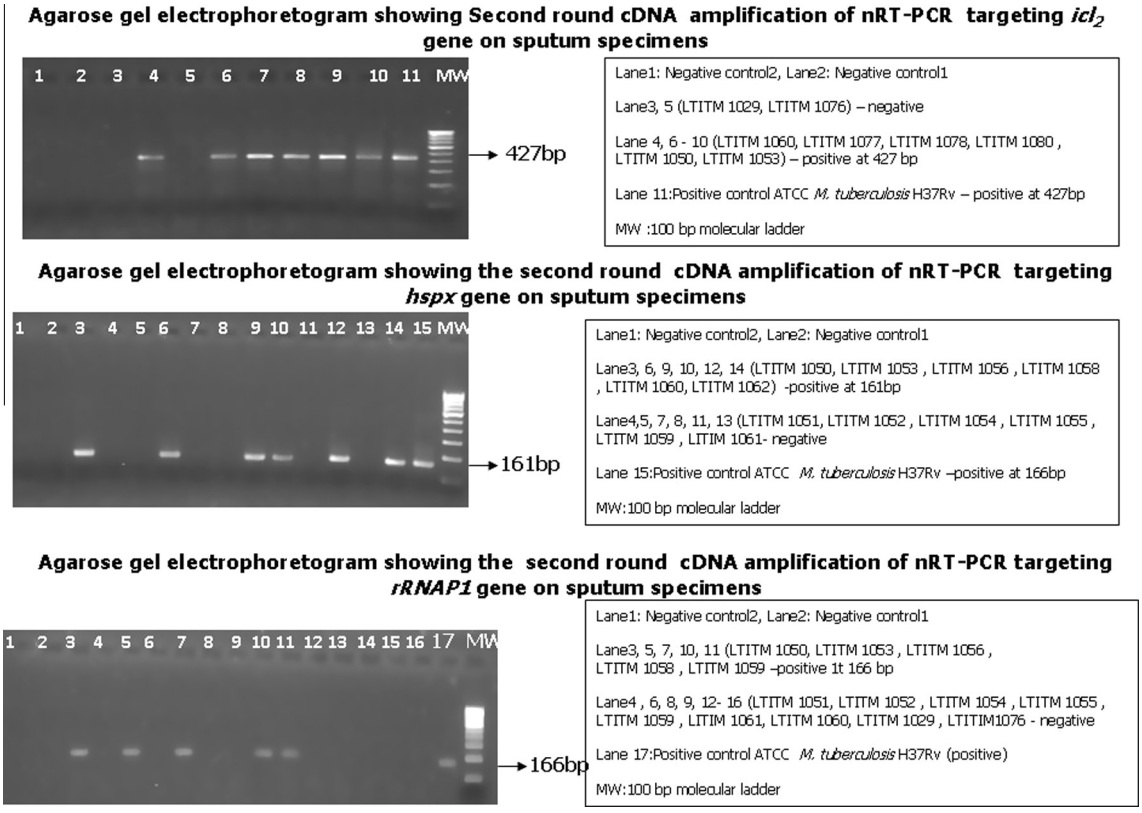

Fig. 1 – Agarose-gel electrophoretograms showing second-round complementary DNA amplification of nested reverse transcriptase–polymerase chain reaction targeting (A) icl_2, (B) *hspx*, and (C) *rRNAP1* genes on sputum specimens. ATCC = American Type Culture Collection.

statistical analysis using the SPSS software (IBM, Chicago, USA) was done for the three nRT–PCRs, and the sensitivity was found to be 36.52% (confidence interval [CI] 27.74–46.01%), 33.93% (CI 25.25–43.48%), and 41.67% (CI 31–52.94%) for the icl_2, *hspx*, and *rRNAP1* genes, respectively. Whereas, when all three nRT–PCRs were analyzed, the sensitivity was increased to 94% with a CI of 82.78–98.62%. Therefore, targeting single gene will not be sufficient to detect viable *M. tuberculosis* from clinical specimens. So, all the three genes (icl_2, *hspx*, and *rRNAP1* genes) must be targeted simultaneously to improve the sensitivity to 100% to detect viable *M. tuberculosis* from the clinical specimens. However, the nRT–PCR negativity in culture-positive clinical specimens in this study was 1.4%. This is one of the significant findings from our study. The 1.4% false negativity in nRT–PCRs resulted in our study may be due to the technical error encountered during collection, transportation, and performance of the experiment. From the results obtained in this study, we can also conclude that, if the standardized nRT–PCRs were positive for two of the three genes (icl_2 and *hspx*, and *rRNAP1* genes), it will provide 100% sensitivity in the detection of actively multiplying *M. tuberculosis* directly from sputum specimens.

Conclusions

In conclusion, nRT–PCRs targeting all the three gene targets optimized in this study need to be applied simultaneously to increase the sensitivity to 100% to detect viable *M. tuberculosis* directly from clinical specimens, which helps in the initiation of an appropriate treatment to the patient. The combinations of indigenously standardized nRT–PCRs are definitely a useful contribution for the development of a rapid molecular diagnostic test for the specific and rapid detection of actively multiplying *M. tuberculosis* genome directly from clinical specimens.

Conflicts of interest

The authors declare no conflicts of interest.

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